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14. ABSTRACT

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Future work will involve the use of protein engineering techniques in order to generate higher efficacy peptides targeting prostate cancer cells followed by their self assembly on quantum dots in order to general multifunctional assemblies for the targeted ablation and imaging of prostate cancer disease.

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1. INTRODUCTION

ADVANCED PROSTATE CANCER DISEASE: Prostate cancer is the most common malignancy affecting men and is the second-leading cause of cancer death in men. Approximately 30,000 deaths occur every year in the United States due to the disease [1]. Localized prostate cancer is generally treated with surgery (radical prostatectomy), radiation therapy, or cryotherapy [2]. However, disease relapse after surgery is a common occurrence mainly due to the outgrowth of minimal residual disease [2]; as high as 48% patients undergoing radical prostatectomy are known to suffer recurrence. It was reported in the National Patterns of Care study, that 35-61% prostate cancer patients receiving radical radiotherapy were left with residual disease [3]. Currently, patients with recurrent, locally advanced, or metastatic prostate cancer are treated by androgen deprivation alone or in combination with local therapy. Although most patients initially respond to androgen deprivation, a large number of patients have disease progression and develop androgen-independent, hormone-refractory prostate cancer [3]. The metastasis of these cells, resistant to conventional therapies such as radiotherapy, is the major cause of death in prostate cancer; the androgen-independent prostate cancer phenotype rapidly leads to mortality and currently there is no effective therapy that significantly increases survival. Clearly, there is significant urgency for the discovery of site-specific therapeutics for residual disease and metastasis in advanced prostate cancer.

PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA): PSMA is a ~100 kDa (750-amino acid) type II membrane glycoprotein with an intracellular segment (amino acids 1–18), a transmembrane domain (amino acids 19–43), and an extensive extracellular domain (amino acids 44–750) [4, 5]. While PSMA is also expressed by cells in the small intestine, proximal renal tubules, and salivary glands, the level of expression in these tissues is 100-1000 fold less than in prostate tissue [6-10]. Alternate splicing of PSMA results in at least three known variants of which PSM' is the most significant. PSMA is an attractive target for site-specific prostate cancer therapy for a variety of reasons. PSMA is over-expressed in human prostate cancer cells and neovasculature rendering a high degree of potential selectivity of a delivered therapeutic to tumor sites [9, 10]. PSMA is abundantly expressed in all stages of prostate cancer disease; the expression of the protein increases in cases of hormone-refractory disease and metastasis, and advanced disease. PSMA is over-expressed in tumors relative to normal cells; PSMA:PSM' ratio was found to be 9-11 in LNCaP cells, 3–6 in prostatic carcinoma, and 0.075–0.45 in normal prostate [11, 12]. Significantly for delivered therapeutics, PSMA and PSMA-antibody complexes have been show to undergo a faster rate of internalization compared to that of PSMA alone [12]. Taken together, these factors make PSMA an attractive target for delivering therapeutics specifically to advanced prostate cancer cells [13, 14].

PEPTIDE AND ANTIBODY BASED THERAPEUTICS: Peptides have been investigated for a number of disease applications [15] including anti-retroviral therapy [16], nucleic acid delivery [17], vaccines [18], antimicrobial therapy [19], and neurodegenerative diseases [20]. Peptides are attractive for anti-cancer therapy [21-24] including prostate cancer [25, 26]. Peptides can be easily synthesized biologically using recombinant means and synthetically using solid-phase techniques, with relatively low production costs. However, in most cases, peptide-based therapeutics lack the high specificites possessed by antibodies. The simultaneous display of multiple peptide copies on a molecular scaffold results in enhanced affinities of the displayed peptide due to ***polyvalency*** therefore, mitigating this limitation. Another limitation is the small molecular size of peptides which results in their rapid clearance by the reticulo-endothelial system and degradation by proteases in the body. Significant effort has therefore been devoted to increasing the half-life of peptides in vivo and modifications such as incorporation of non-natural amino acids have been employed to limit proteolysis. Antibody-based constructs have emerged as important therapeutics in several human malignancies including breast cancer, lymphoma, colorectal cancer, and lung cancer [22, 27]. Although unmodified antibodies show some therapeutic benefit, antibodies have been mainly used to specifically target cytotoxic agents including chemotherapeutic drugs, radionuclides, enzymes, and cytokines specifically to cancer cells with high efficacies.

Prostate cancer is appropriate target for antibody-based therapies for a number of reasons: (1) the prostate is a non-essential organ which allows organ- or tissue-specific antigen based targeting, (2). The common regions of prostate cancer metastasis, bone and lymph nodes, receive high levels of circulating antibodies, and (3) the small size of prostate cancer metastasis implies easier antigen access and higher penetration of antibodies. Consequently, antibody-based constructs are being investigated in prostate cancer therapy.

NANOMEDICINE: Nanomedicine marries the potential of nanotechnology with the rapidly progressing understanding of molecular and cellular biology, resulting in potential therapeutic and diagnostic benefits. A number of nanoparticles and nanomaterials are being investigated for their potential application in biomedical research [28]. Gold and iron oxide nanoparticles have been investigated in potential biosensor and Magnetic Resonance Imaging (MRI). In addition to inorganic nanoparticles, biodegradable polymeric nanocapsules, dendrimeric molecules, protein cage architectures, liposomes, and polyplexes have been investigated for a variety of drug delivery applications. Quantum dots (QDs) are colloidal semiconductor nanocrystals with size-dependent absorption and emission properties[29]. QDs are available in a large number of well-separated 'colors' all of which can be excited by a single wavelength. A wide variety of conjugation chemistries have been explored for either self-assembling or conjugating peptides oligonucleotides, and antibodies bioactive molecules on QDs for live-cell and in vivo applications [29-34]. However, due to the embryonic stage of research in this area, the potential of QDs has not yet been fully realized in cancer therapy.

New developments in the field will have a significant impact on cancer diagnostics and therapeutics.

This study describes the development of targeted therapies for the advanced (i.e. androgen independent and radiation-insensitive) prostate cancer phenotype based on the induction of apoptosis in cells using mitochondria depolarizing peptides (MDPs). Two parallel strategies are being investigated for the site specific delivery of MDPs to prostate cancer cells: 1). Quantum dot-polypeptide nanoassemblies and 2).Antibody-MDP conjugates. The hypothesis/ rationale/ purpose of the proposed research are described below.

2. HYPOTHESIS/RATIONALE/PURPOSE

The transformation of androgen-dependent disease to highly tumorigenic, metastatic, and androgen independent phenotype is a result of the accumulation of significant genetic changes. For example, overexpression of anti-apoptotic proteins (e.g. Bcl-2) leads to aggressive survival in advanced prostate cancer cells. Treatments that overcome/bypass these phenotypic changes and enhance apoptosis of cancer cells are attractive therapeutic strategies. The anti-microbial peptide KLA59 (single letter sequence: KLAKLAKKLAKLAK) has been shown to induce apoptosis in cancer cells due to its ability to depolarize mitochondrial membranes [35]. The large negative potential (-180mV) across the mitochondrial membranes [36] is the driving force that results in localization of cationic amphipathic peptides in the mitochondria. In time, the localization of the peptide results in the depolarization of the membrane, membrane permeability transition, and release of mitochondrial contents into the cytoplasm. The release of pro-apoptotic proteins from the mitochondria including, cytochrome-c, SMAC, and AIF ultimately results in apoptosis following depolarization. Using MDPs as cancer therapeutics is attractive because the strategy acts directly on the mitochondria and avoids the effects of anti-apoptotic proteins upstream of the organelle. The inefficient delivery of therapeutics to tumor sites remains the primary obstacle in prostate cancer therapy. In particular, the preservation and damage-prevention to underlying and collateral organs such as the rectum, bladder and the urethra is important and the damage caused to nearby tissues due to inefficient targeting is cause for concern. Thus, while the use of MDPs (e.g. KLA) is attractive, strategies that direct these peptides to the diseased tissue and individual prostate cancer cells need to be developed. The hypotheses of the proposed research are summarized below:

A: PSMA is an appropriate target in advanced prostate cancer therapy. PSMA is over-expressed in prostate cancer cells and neovasculature of malignant neoplasms rendering a high degree of selectivity of a delivered therapeutic to tumor sites.

B: Antibody and peptide mediated delivery of MDPs targeted specifically to prostate cancer cells will result in the selective induction of apoptosis in these cells. The identification of cyclic peptides with micromolar (μM) binding affinities to PSMA63 (PSMA-targeting peptides; PTPs) is an opportunity to develop peptide-based targeting strategies in prostate cancer therapy. In addition, the nanomolar (nM) affinities of anti-PSMA monoclonal antibodies make it an attractive vehicle for delivering MDPs specifically to prostate cancer cells.

C: Oligomerization of PSMA-targeting peptides using molecular self-assembly can result in enhanced targeting of the PSMA. The oligomeric display of peptide epitopes using self-assembling coiled coil peptides has been demonstrated to result in mutants with higher binding affinities [37]. Using a similar approach, PTP epitopes will be displayed on oligomeric (dimeric, trimeric, and tetrameric) α-helical coiled-coil peptides as a PTP-coiled coil fusion peptide which possesses high binding affinities to the PSMA receptor.

D: The self-assembly of multiple PTP oligomers and MDPs with quantum dots will lead to multi-Dunctional constructs. It is hypothesized that the simultaneous loading of PTP oligomers and MDPs on quantum dots (QDs; 1-10nm diameter) can lead to a 'multi-functional' QD-polypeptide constructs with targeting (PTP), apoptotic (MDP) and imaging (QD) capabilities on a single platform.
The apoptotic efficacies of QD-polypeptide assemblies and MLN591-MDP conjugates will be evaluated using different prostate cancer cell lines. Promising leads from these experiments will be investigated in vivo using orthotopically implanted prostate cancer tumors in mice. This research will lead to valuable pre-clinical information and increase the array of site-specific therapeutics for advanced prostate cancer.

SPECIFIC AIMS

1. Generation and Characterization of Apoptosis-Inducing Quantum Dot-Polypeptide Assemblies.
2. Generation and Characterization of Apoptosis-Inducing Antibody-MDP Conjugates.
3. Parallel, Cell-Based In-Vitro Evaluation of QD-Polypeptide and MLN591-KLA Conjugates.
4. In-Vivo Evaluation of QD-Polypeptide and MLN591-KLA Conjugates.

3. KEY RESEARCH ACCOMPLISHMENTS

A significant amount of progress has been made with respect to the work described in the above proposal. We have evaluated cationic amphipathic peptide based fusion peptides (Specific Aim 1) and immunoconjugates (Specific Aim 2) for the targeted ablation of prostate cancer cells. These results have resulted in a manuscript published recently in the journal Cancer Research (see Appendix 1). Secondly, we have investigated the role of the Prostate-Specific Membrane Antigen (PSMA) in the differential intracellular sorting of fluorescent quantum dots by prostate cancer cells (Specific Aim 3). A manuscript relating to these results in currently in preparation. The experimental progress to date is described in Section B.

Future work will involve the use of protein engineering techniques in order to generate higher efficacy peptides targeting prostate cancer cells followed by their self assembly on quantum dots in order to general multifunctional assemblies for the targeted ablation and imaging of prostate cancer disease.

4. REPORTABLE OUTCOMES

A. IN VITRO EVALUATION OF FUSION PEPTIDES AND IMMUNOCONJUGATES AS TARGETED THERAPEUTICS FOR ADVANCED PROSTATE CANCER DISEASE (APPENDIX 1): We exploited the over-expression of the Prostate-Specific Membrane Antigen (PSMA) on the surface of prostate cancer cells as means to selectively deliver the mitochondria-depolarizing peptide KLA (single letter sequence KLAKLAKKLAKLAK) to these cells. We investigated the potency and mechanism of cell death in human prostate cancer cell lines treated with untargeted, peptide-targeted and monoclonal antibody-targeted MDP constructs. These results, described below, are reported in a manuscript recently accepted for publication in the journal ***Cancer Research*** **(APPENDIX 1)**.

The untargeted KLA peptide induced significant non-specific cell death (greater than 70% loss in viability) in both PC-3 and LNCaP cells at concentrations greater than 70μM. However, while low concentrations of KLA induced death in LNCaP cells, concentrations lower than 35μM had no effect on PC-3 cells. Addition of the PSMA-targeting peptide (PTP) element to KLA resulted in increased potency of the resulting fusion peptide PTP-KLA in LNCaP cells, but not PC-3 cells. To evaluate the mechanism by which the PTP element enhances the potency of KLA, we performed competitive inhibition experiments to determine if PTP-KLA is internalized via specific interaction with the PSMA receptor. Competitive inhibition experiments indicated

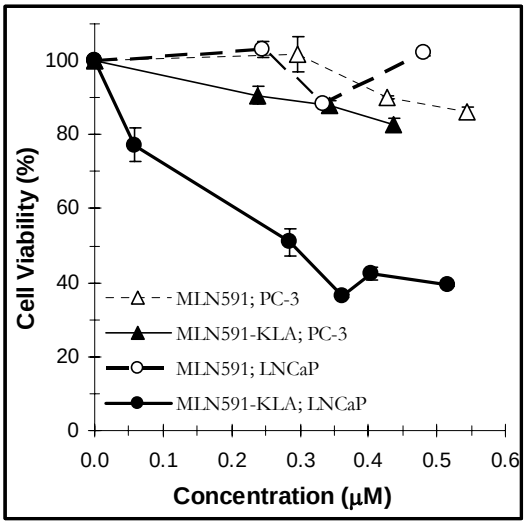

FIGURE 1. Comparison of MLN591-KLA activity on LNCaP and PC-3 cells treated for 72h with different concentrations of MLN591 and MLN591-KLA (3-5 KLA molecules per MLN591 molecule). The lines connecting the data points are for visualization only.

inhibition of PTP-KLA activity by PTP; while 25 μM PTP completely reversed the cytotoxic effects of 22 μM PTP-KLA on LNCaP cells, less inhibition was observed upon raising the PTP-KLA concentration to 30 μM. We verified that PTP alone was not cytotoxic under these conditions. Pre-incubation with 500 nM anti-PSMA antibody (MLN591) also resulted in a reduction in cell death induced by PTP-KLA.

Conjugation of KLA to anti-PSMA antibody MLN591 enhances its potency in LNCaP cells: In addition to the fusion peptides described above, we investigated antibody (MLN591)-KLA conjugates as potential site-specific therapeutics for prostate cancer. Briefly, primary amines on the MLN591 antibody were reacted with the hetero-bifunctional cross-linker SPDP to obtain MLN591-SPDP conjugates. The KLA peptide was then conjugated to the activated MLN591 antibody via the thiol group of the C-terminal cysteine on the peptide. A ratio of 3-5 peptide molecules per antibody molecule was consistently obtained using this method.

The antibody-KLA conjugate was significantly more cytotoxic to LNCaP cells than KLA or PTP-KLA. Figure 1 compares the cell viability of LNCaP and PC-3 cells treated with different concentrations of MLN591-KLA for 72 h; no loss of cell viability was observed in either cell line at 24 h. The MLN591-KLA conjugate had no effect on PC-3 cell viability but induced significant cell death in LNCaP cells, indicating selectivity of the conjugate towards PSMA-expressing LNCaP cells (Figure 1). Unconjugated MLN591 did not result in a loss of cell viability in either PC-3 or LNCaP cells. Furthermore, we verified that LNCaP cell viability was unaffected by treatment with equivalent concentrations of either KLA or PTP-KLA for 72 h (not shown).

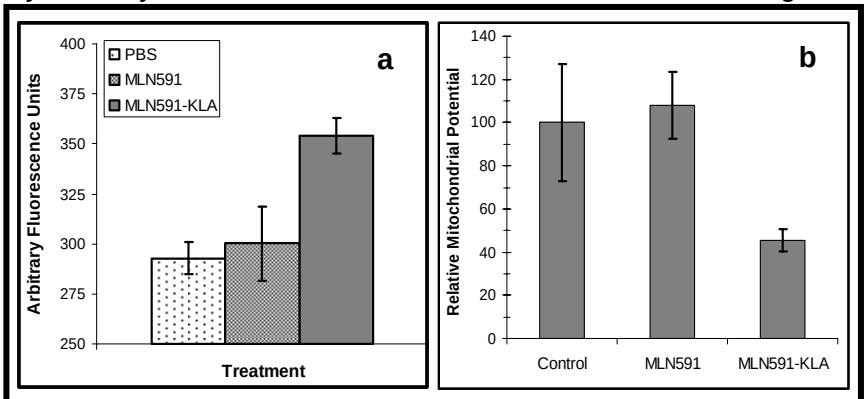

FIGURE 2 a. Caspase activation in LNCaP cells treated with PBS, 0.4μM MLN591 or 0.4μM MLN591-KLA determined by the homogeneous caspases assay. b. Comparison of relative mitochondrial potential in untreated, 0.4μM MLN591, and 0.4μM MLN591-KLA treated cells. Cells were treated with JC-1 for 45 min and the fluorescence intensities were measured. The ratio of the fluorescence intensity at 590nm to that at 510nm was measured in each case (excitation: 485nm). Relative mitochondrial potential was determined by comparing the ratio of the fluorescence intensities with that of the control (untreated sample).

Mechanisms of Cell Death: PTP-KLA MLN591-KLA induces apoptosis in LNCaP cells: Flow cytometry analysis of propidium iodide stained LNCaP cells after MLN591-KLA treatment indicated that a significant fraction of the cells (>80%) were apoptotic, as indicated by a sub-G0 population analysis (not shown). In contrast, equivalent concentrations of the MLN591 antibody did not induce apoptosis in these cells (not shown). The homogeneous caspases assay was carried out to investigate caspase activation in LNCaP cells upon incubation with MLN591-KLA for 72 h. Figure 2a shows caspase activation when LNCaP cells were treated with MLN591-KLA while those treated with MLN591 exhibited caspase activity comparable to cells treated with PBS. The assay detects caspases 2, 3, and 7 indicating a role for these enzymes in MLN591-KLA mediated cell death. In addition, LNCaP cells were pre-incubated with 100 μM of the pan-caspase inhibitor z-VAD-Fmk for 2 h before treatment with the MLN591-KLA conjugate. Inhibition of caspase activity with z-VAD-Fmk increased LNCaP viability (not shown), supporting a role for the caspase-mediated apoptotic pathway in MLN591-KLA-induced cell death. Finally, the MLN591-KLA conjugate induced mitochondrial depolarization in LNCaP cells which is consistent with the proposed mechanism for cell death induced by the cationic amphipathic peptide KLA (Figure 2b). The mitochondrial integrity of LNCaP cells treated with the unconjugated MLN591 antibody was similar to that of the control. **Comparison of KLA, PTP-KLA and**

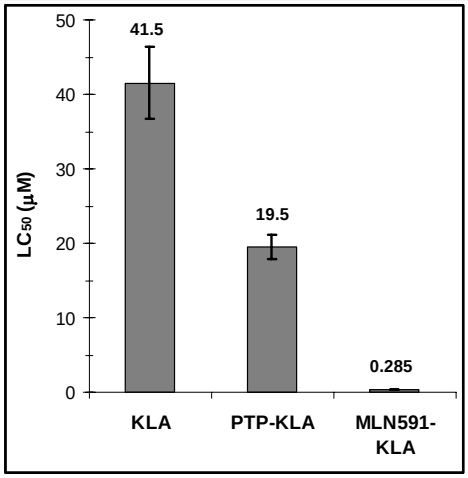

FIGURE 3. Comparison of the in vitro LC_{50} values of untargeted, peptide-targeted and antibody targeted fusions / conjugates of KLA with LNCaP cells.

MLN591-KLA: Dose response studies were carried out to determine the in vitro LC_{50} values for KLA, PTP-KLA and MLN591-KLA in LNCaP cells. The LC_{50} value of the MLN591-KLA conjugate for LNCaP cells was 0.28 μM which is approximately 70-fold lower than that of PTP-KLA (19 μM) and more than two orders of magnitude lower than that of KLA (41 μM), indicating that the anti-PSMA antibody significantly enhances the potency of the KLA peptide (Figure 3). In addition, while PTP-KLA and KLA induced oncotic death in LNCaP cells, MLN591-KLA induced caspase-mediated apoptotic death.

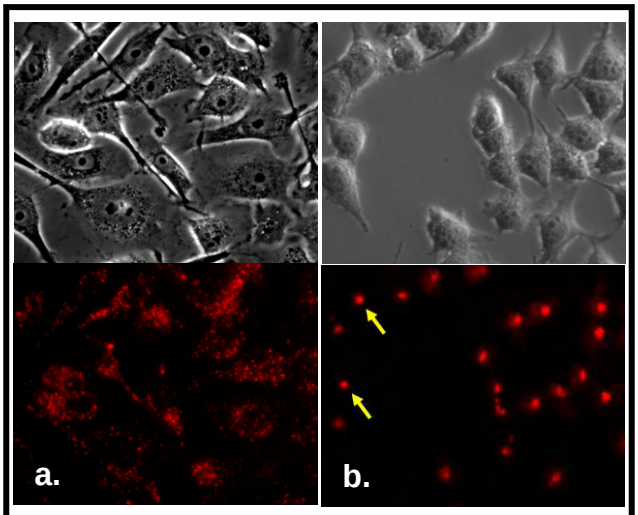

FIGURE 4. Differential intracellular sorting of quantum dots in PSMA non-expressing PC3 (a) and PSMA-expressing PC3-PSMA prostate cancer cells.

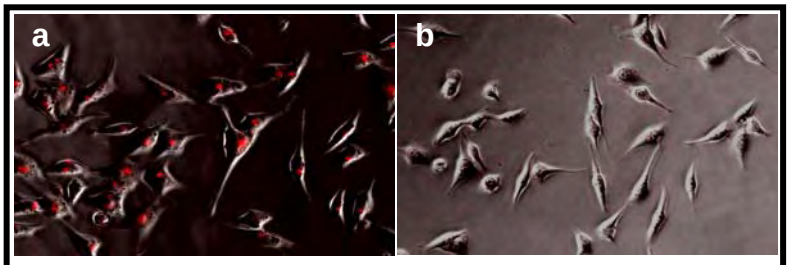

FIGURE 5. PC3-PSMA cells internalize QDs via clathrin-mediated endocytosis (a). Cells treated with the clathrin inhibitor chlorpromazine do not internalize QDs (b).

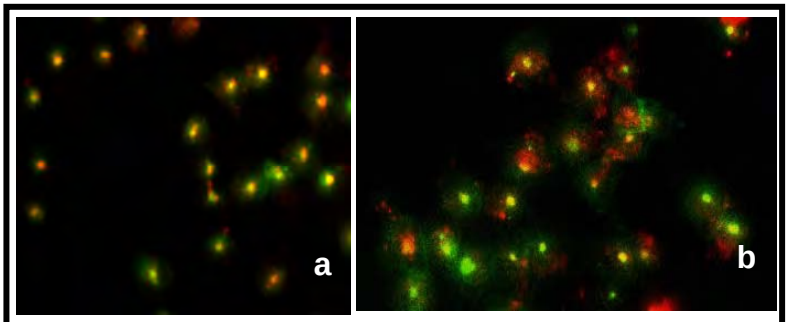

FIGURE 6. (a) Colocalization of QDs (red) with AlexaFluor 488-transferrin (green) and (b) QDs (red) with FITC-labeled anti-PSMA antibody is seen as yellow in both cases indicating the localization of QDs in recycling endosomal compartments in PC3-PSMA cells.

B. INTRACELLULAR SORTING OF QUANTUM DOTS BY PROSTATE CANCER CELL LINES. Guided by our interest in the development of nanoscale therapeutics, we **(Rege)** evaluated the uptake and intracellular trafficking of quantum dots (QDs) by prostate cancer cell lines. Figure 4 shows the differential uptake of 655nm carboxylated QDs by PC3 and PC3-PSMA cells (PC3 cells transduced to stably express the PSMA receptor on the surface) as visualized by fluorescence microscopy. While QDs showed a punctate profile indicating lysosomal localization in PC3 cells (Figure 4a), they localized to a single location in PC3-PSMA cells (Figure 4b). Further investigation indicated that although lipid raft disruption (using methyl-β-cyclodextrin based cholesterol extraction) did not alter QD uptake (not shown), clathrin disruption (using chlorpromazine) completely inhibited QD uptake by both cells (Figure 5 a, b). QDs co-localized with AlexaFluor 488-Transferrin in PC3-PSMA cells (Figure 6a) indicating the localization of these nanoparticles in the recycling endosomal compartment (REC). Interestingly, QDs also colocalized with PSMA as determined by colocalization with FITC-labeled anti-PSMA antibody (Figure 6b). Taken together, our results indicate the differential sorting of QDs in prostate cancer cells: the absence of cell-surface PSMA resulted in clathrin-mediated lysozomal localization in PC3 cells; the presence of PSMA resulted in the localization of QDs in the REC. We hypothesize that the presence of cell-surface PSMA plays an active role in the trafficking of the carboxylated QDs to the REC in PC3-PSMA cells; this is corroborated by the colocalization of QDs and anti-PSMA antibody in RECs.

5. CONCLUSIONS

We have designed, generated, and evaluated targeted fusion peptides and immunoconjugates based on the cationic amphipathic peptide KLA as potential targeted therapeutics for prostate cancer. Our results indicate that antibody-KLA conjugates have promise as potential therapeutics and warrant further investigation. We also investigated the mechanism of cell death induced by these molecular therapeutics. In cases where cationic amphipathic peptides are used, the affinity of the targeting sequence to the receptor must be significantly higher than the membrane-permeabilizing affinity of the lytic peptide to minimize nonspecific death. Thus, whereas lower concentrations of MLN591-KLA are sufficient to induce apoptosis in LNCaP cells due to the enhanced affinity of MLN591 antibody to the receptor, further engineering of peptides targeting the PSMA is required in order for them to be useful in potential therapeutic applications.

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Research Article

Amphipathic Peptide-Based Fusion Peptides and Immunoconjugates for the Targeted Ablation of Prostate Cancer Cells

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Abstract

We describe the design, generation, and *in vitro* evaluation of targeted amphipathic fusion peptides and immunoconjugates for the ablation of prostate cancer cells. The overexpression of the prostate-specific membrane antigen (PSMA) was exploited as means to specifically deliver cytotoxic peptides to prostate cancer cells. Cationic amphipathic lytic peptides were chosen as cytotoxic agents due to their ability to depolarize mitochondrial membranes and induce apoptosis. Specific delivery of the lytic peptide was facilitated by PSMA-targeting peptides and antibodies. Our results indicate that although the use of PSMA-targeted peptides only modestly enhanced the cytotoxic activity of the lytic peptide, peptide-antibody conjugates were two orders of magnitude more potent than untargeted peptide. In addition to quantifying the cytotoxic activities of the individual constructs, we also investigated the mechanisms of cell death induced by the fusion peptides and immunoconjugates. Although fusion peptides induced oncotic/necrotic death in cells, treatment with immunoconjugates resulted in apoptotic death. In summary, immunoconjugates based on lytic peptides are a promising class of therapeutics for prostate cancer therapy and warrant further investigation. [Cancer Res 2007;67(13):6368–75]

Introduction

Prostate cancer is the most commonly diagnosed malignancy and is the second leading cause of cancer death in men; ~30,000 deaths occur every year in the United States due to the disease (1, 2). Localized prostate cancer is generally treated with surgery (radical prostatectomy), radiation therapy, or cryotherapy (3). However, disease relapse after surgery is a common occurrence, mainly due to the outgrowth of minimal residual disease (3). Currently, patients with recurrent, locally advanced, or metastatic prostate cancer are treated by androgen deprivation alone or in combination with local therapy. Although most patients initially respond to androgen deprivation, a large fraction of patients suffer disease progression and develop androgen-independent, hormone-refractory prostate cancer. The metastasis of these androgen-independent cells, which are also resistant to conventional therapies such as radiotherapy, is the major cause of death in prostate cancer. Treatment options in these cases are fairly limited, and most available treatments rely on chemotherapeutic drugs (4), which have variable efficacies and severe side effects. As a result, there is a need to develop site-specific (targeted) therapeutics for the treatment of residual and metastatic prostate cancer.

The prostate-specific membrane antigen (PSMA) is a type II membrane glycoprotein with an intracellular segment (amino acids 1–18), a transmembrane domain (amino acids 19–43), and an extensive extracellular domain (amino acids 44–750; refs. 5, 6). PSMA is abundantly expressed in all stages of prostate cancer disease; the expression of the protein increases in cases of hormone-refractory disease, metastasis, and advanced disease, making it an attractive target for site-specific prostate cancer therapy (7–11). Although PSMA is also expressed by cells in the small intestine, proximal renal tubules, and salivary glands, the level of expression in these tissues is 100- to 1,000-fold less than in prostate tissue, rendering a high degree of potential selectivity for site-specific therapeutic agents (5, 7–11). Furthermore, PSMA is also expressed in the neovasculature of a wide variety of malignant neoplasms, including prostate, lung, colon, breast, and others, but not in normal vascular endothelium (12). Significantly, PSMA and PSMA-antibody complexes have been shown to undergo internalization through clathrin-coated pits (12, 13), indicating that the receptor can be used for intracellular delivery of therapeutics. Consequently, different strategies, including antibodies and antibody-conjugated toxins, have been investigated for targeting PSMA-expressing prostate cancer cells (14–16).

Peptides/fusion peptides are increasingly gaining popularity as therapeutic agents for a variety of applications (17), including antiretroviral therapy (18), nucleic acid delivery (19), tumor vaccines (20), antimicrobial therapy (21), and neurodegenerative diseases (22). Peptide-based therapeutics are also attractive candidates for anticancer therapy (23–26), including prostate cancer (27, 28). Peptide therapeutics are easily produced using either recombinant or solid-phase chemical synthesis techniques and are generally less expensive than antibody-based therapeutics. In addition, as opposed to immunoconjugates, the small size of fusion peptides makes it possible to deliver them using routes other than intraveneous injection (17). However, the use of peptides as targeting agents in site-specific delivery is often limited by their low binding affinities to their respective targets compared with those of antibodies. Furthermore, their small molecular size results in rapid clearance by the reticuloendothelial system and degradation by proteases in the body.

The antimicrobial peptide KLA (ref. 29; single-letter amino acid sequence: KLAKLAKKLAKLAK) has been shown to induce apoptosis in cancer cells, presumably due to its ability to depolarize mitochondrial membranes (23). The large negative potential (−180 mV) across the mitochondrial membrane (30) promotes the accumulation of the positively charged amphipathic peptide at its surface. In time, this localization results in membrane depolarization, a membrane permeability transition, and the release of mitochondrial contents into the cytoplasm. The release of proapoptotic proteins from the mitochondria, including cytochrome *c*, second mitochondrial derived activator of caspase, and apoptosis-inducing factor (AIF), ultimately results in apoptosis (31).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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We describe the design, generation, and evaluation of PSMA-targeted fusion peptides and antibody-peptide conjugates as site-specific therapeutics for advanced prostate cancer disease. The recent identification of peptides targeting the extracellular portion of PSMA (32) enables the development of peptide-based strategies for targeting malignant prostatic tissue. We designed a fusion peptide composed of a PSMA-targeting peptide (PTP) and the cationic amphipathic peptide KLA, and investigated the *in vitro* efficacy, selectivity, and mechanism of cell death induced by the PTP-KLA fusion peptide in LNCaP and PC-3 human prostate cancer cell lines. In addition, we explored the *in vitro* efficacy and mechanisms of cell death induced by anti-PSMA antibody-KLA immunoconjugates. Our results indicate that the although the PTP-KLA fusion peptide possesses only moderate potency, the antibody-KLA conjugate potently and selectively induces cell death in PSMA-expressing LNCaP cells, thus meriting further investigation as a potential therapeutic for prostate cancer.

Materials and Methods

Cell lines and cell culture. Human prostate cancer cell lines LNCaP (PSMA positive) and PC-3 (PSMA negative) were obtained from the American Type Culture Collection (ATCC). LNCaP cells were grown in RPMI 1640 (ATCC) + 10% fetal bovine serum (FBS; Invitrogen Corp.) + 1% penicillin/streptomycin (Invitrogen). PC-3 cells were cultured in F-12K media (Invitrogen) containing 10% FBS and 1% penicillin/streptomycin. The cells were cultured at 37°C in a 5% CO_2 incubator. All chemicals were purchased from Sigma unless otherwise specified.

Fusion peptides. The following peptides were purchased from the Tufts University Core Facility, Medford, MA:

1. KLA: HHHHHH**KLAKLAKKLAKLAK**C (the cationic amphipathic, lytic peptide sequence is in bold font)
2. PTP: **CQKHHNYLC**GGHHHHH (the PTP, ref. 32, is in bold font)
3. PTP-KLA: CQKHHNYLCGGKLAKLAKKLAKLAKHHHHH

The peptides were chemically synthesized and purified by high-performance liquid chromatography. The molecular weights of the purified fractions were verified by mass spectrometry, and those fractions (>98% purity) that corresponded to the correct peptide molecular weight were lyophilized for use in experiments. Stock solutions of the lyophilized peptides were prepared in PBS, aliquoted, and stored at −20°C. Peptide concentrations were calculated using the ninhydrin assay (33). For cyclization of PTP (via formation of a disulfide bond between C1 and C9), 0.1 mg/mL peptide in deionized water was oxidized in air at room temperature for 4 days. The formation of the disulfide bond was verified using Ellman's reagent (Pierce Biotechnology) and cysteine standards as described in the vendor's protocol. The peptide was then lyophilized and resuspended in sterile PBS. All peptides were >98% pure and were sterile filtered (polyethersulfone 0.2-μm syringe filter, Nalgene) immediately before use.

Antibody-KLA conjugation. The anti-PSMA monoclonal antibody, MLN591, was a gift from Millennium Pharmaceuticals Inc. The MLN591 antibody was dialyzed thrice against PBS using a 3,500 molecular weight cutoff float-a-lyzer membrane (Spectrum Labs) at 4°C. The MLN591 antibody was then conjugated with the KLA peptide using an *N*-succinidyl-3-(2-pyridylothio)propionate (SPDP) linker (Pierce). Briefly, MLN591 (1 mg/mL; 1 mL) was reacted with 20 μL of 5 mmol/L SPDP dissolved in DMSO at room temperature for 15 min with shaking. Unreacted SPDP was removed by gel filtration using a Bio-Rad 10G desalting column (3 mL, exclusion limit 3 kDa) under gravity. Fractions (1 mL) were collected by flowing PBS through the column; the fractions were concentrated using a spin vacuum system and pooled to make a final volume of 1 mL. To verify the conjugation of the SPDP linker to the antibody, 100 μL of the pooled fractions were diluted to 1 mL and reacted with 10 μL DTT (15 mg/mL in PBS) for 15 min. DTT treatment results in release of for pyridine-2-thione which can be monitored at 343 nm and is an indicator of the conjugation of the SPDP linker to MLN591. The number of SPDP molecules conjugated per MLN591 molecule was determined using the SPDP conjugation protocol (Pierce). The MLN591-SPDP conjugate was then reacted with 300 μL of KLA peptide (200–300 μmol/L in PBS) at room temperature. Before the reaction, 400 μL of KLA was mixed with 10 mg of Reductacryl (EMD Biosciences Inc.) for 10 min to reduce cysteine thiols and enable conjugation to the MLN591-SPDP conjugate. Following the reduction step, 300 μL of the reduced peptide solution was recovered from the resin by centrifugation at 11,000 × *g* for 10 min. The kinetics of the MLN591-KLA conjugation reaction were monitored using absorbance at 343 nm; in all cases, the reaction was complete within 3 to 4 h. The number of KLA molecules conjugated to the MLN591 antibody was estimated as described previously. Using this protocol, we were consistently able to conjugate three to five molecules of KLA per molecule of MLN591. Finally, the MLN591-KLA reaction mixture was dialyzed thrice against PBS at 4°C to remove unreacted KLA, sterile-filtered through a 0.22-μm syringe filter, and stored at 4°C for further use.

Cytotoxicity assays. LNCaP and PC-3 cells were plated in 24-well cell culture plates (Corning Inc.) at a density of 125,000 cells per well and allowed to attach for 24 h. Cells were incubated with different concentrations of KLA, PTP-KLA, and MLN591-KLA, followed by an analysis for viability. Briefly, following individual treatments, cells were treated with 100 μL calcein AM-ethidium homodimer stock solutions (Invitrogen; final concentrations: calcein, 2 μmol/L; ethidium homodimer, 4 μmol/L) for 30 min, centrifuged (Beckman GS-6R) at 4°C, washed with 1 mL PBS, centrifuged again at 4°C, resuspended in 1 mL PBS, and immediately analyzed using flow cytometry (Beckman Coulter Epics Altra). A total of 10,000 events were recorded for each sample, and cells that stained red (ethidium homodimer positive) were considered dead. Cell viability and proliferation was also analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (ATCC) using absorbance at 570 nm. For the evaluation of antibody-based conjugates, cells were treated with buffer-exchanged MLN591 (MLN591 dialyzed thrice against PBS at 4°C), MLN591-KLA, or equivalent amounts of PBS (control) for 72 h, after which they were harvested and analyzed for viability as described above. All experiments were carried out at least in triplicate.

Apoptosis assays. For determination of the sub-G_0 phase population, LNCaP cells were treated with the peptides and the antibody conjugate as described above, centrifuged, fixed in 70% ethanol for 1 h at room temperature, washed twice with PBS, and stained with propidium iodide for 45 min. Cells were washed once with PBS and analyzed by flow cytometry; a total of 10,000 events were evaluated. Apoptosis/oncosis was also investigated using the annexin V–FLUOS/propidium iodide staining kit (Roche Applied Sciences). Briefly, following treatment, LNCaP cells were washed with 500 μL PBS and 20 μL annexin V–FLUOS, and propidium iodide were dissolved in the incubation buffer, and cells were incubated with 50 μL of this stock solution for 30 min. Cells were then washed with 500 μL PBS and analyzed by fluorescence microscopy (Zeiss Axiovert 200M, Carl Zeiss MicroImaging, Inc.). Cells stained with annexin V–FLUOS alone (green fluorescence) were considered apoptotic, whereas those that stained both green and red were considered oncotic.

To probe the possible involvement of the caspase-mediated apoptotic pathway, cells were treated with the pan-caspase inhibitor z-VAD-Fmk (100 μmol/L; R&D Systems) for 2 h before treatment with KLA and PTP-KLA peptides and MLN591-KLA conjugates. Cell viability was determined at 24 h for the peptides and at 72 h for MLN591-KLA immunoconjugates as described above; viability of cells treated with the inhibitor was compared with untreated cells to determine the involvement of the caspase-mediated apoptotic pathway. The homogeneous caspase assay (Roche Applied Sciences) was used to investigate caspase activation in LNCaP cells treated with immunoconjugates, following protocols recommended by the vendor. The release of rhodamine, which is an indicator of caspase activity in the assay, was detected by fluorescence, and fluorescence intensity was used to compare caspase activation across different treatments.

Mitochondrial depolarization. The JC-1 dye (Invitrogen) forms red-fluorescent J-aggregates upon localization in healthy mitochondria, whereas the monomeric form of the dye fluoresces green in the cytoplasm (34).

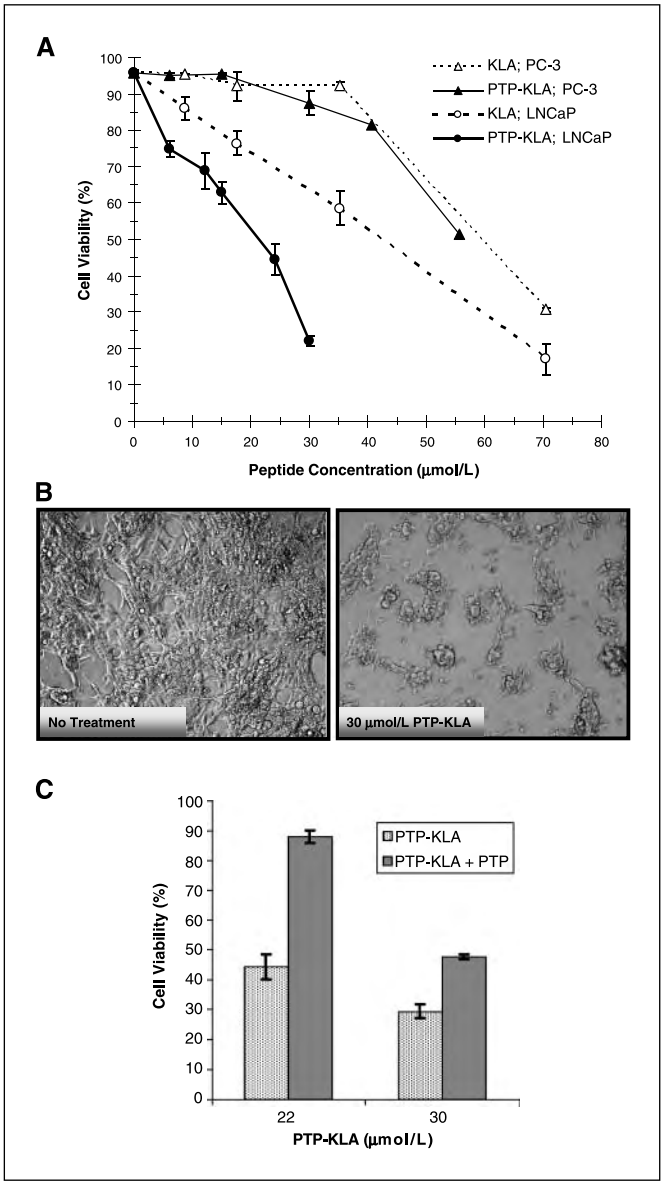

Figure 1. *A,* prostate cancer cell viability as a function of peptide concentration. Cells (125,000 per well) were treated with different concentrations of the peptides for 24 h, following which cell viability was determined and compared with the control (cells treated with equal volume of PBS). △, PC-3 cells treated with KLA; ▲, PC-3 cells treated with PTP-KLA; ○, LNCaP cells treated with KLA; ●, LNCaP cells treated with PTP-KLA. The lines connecting the data points are for visualization only. *B,* Representative morphologies of untreated LNCaP cells and LNCaP cells treated with 30 μmol/L PTP-KLA for 24 h. *C,* competitive inhibition of PTP-KLA by the PSMA targeting peptide (PTP; see Materials and Methods for peptide sequences). LNCaP cells were pretreated with PTP (25 μmol/L) for 2 h or left untreated, followed by treatment with PTP-KLA (22 or 30 μmol/L) for 24 h.

LNCaP cells were treated with the peptides or the antibody-KLA conjugate as described above and stained with JC-1 (25 μmol/L) in the dark for 45 min at 37°C, washed with 1 mL PBS, resuspended in 1 mL PBS, and analyzed by flow cytometry. The decrease in the ratio of red to green fluorescence was used to determine relative mitochondrial depolarization. In addition to flow cytometry, the fluorescence intensities [excitation: 485 nm; emission: 510 nm (green); emission: 590 nm (red)] were also measured using a spectrofluorometer (Molecular Devices fmax; Molecular Devices Corp.).

Statistical analysis. Results are presented as mean ± 1 SD of at least triplicate experiments.

Results

Evaluation of KLA and PTP-KLA Fusion Peptides

PTP enhances the cytotoxic efficacy of KLA in PSMA-expressing LNCaP cells. Although well documented in literature (6, 35), we first verified the presence and absence of PSMA on the surface of LNCaP and PC-3 cells, respectively, using immunofluorescence (data not shown). The cationic amphipathic untargeted KLA peptide induced significant nonspecific cell death (> 70% loss in viability) in both PC-3 and LNCaP cells at concentrations >70 μmol/L (Fig. 1*A*). However, although lower KLA concentrations (35 μmol/L) induced death in LNCaP cells, no PC-3 cell death was observed under these conditions. Addition of the PSMA-targeting peptide (PTP) to KLA increased its potency in LNCaP cells, but not PC-3 cells (Fig. 1*A*). The loss of viability in LNCaP cells was accompanied by characteristic cell detachment and clumping (Fig. 1*B*).

To evaluate the mechanism by which PTP enhances the potency of KLA, competitive inhibition experiments were carried out to determine if PTP-KLA is internalized via specific interaction with the PSMA receptor. Competitive inhibition experiments indicated the inhibition of PTP-KLA activity by PTP (Fig. 1*C*). Although 25 μmol/L PTP completely reversed the cytotoxic effects of 22 μmol/L PTP-KLA on LNCaP cells, less inhibition was observed upon raising the PTP-KLA concentration to 30 μmol/L. We verified that PTP alone was not cytotoxic under these conditions (data not shown). Preincubation with 500 nmol/L anti-PSMA antibody (MLN591) also resulted in a reduction in cell death induced by PTP-KLA (data not shown).

PTP-KLA induces oncotic/necrotic death in LNCaP cells. We then investigated the mechanism of cell death (apoptosis/oncosis) induced by the two peptides in LNCaP cells. Preincubation with the pan-caspase inhibitor z-VAD-Fmk (100 μmol/L) did not inhibit LNCaP cell death after treatment with 30 μmol/L PTP-KLA (Fig. 2*A*), indicating that the caspase-mediated apoptotic pathway was not involved in cell death and further validating the observation that oncosis was the primary mode of cell death. In addition, LNCaP cells were treated with KLA and PTP-KLA and stained with annexin V–FLUOS and propidium iodide to differentiate between purely apoptotic populations from a mixture of late apoptotic/oncotic ones. Cells treated with 30 μmol/L PTP-KLA stained positive for propidium iodide, indicating that the mode of cell death induced by this concentration of the peptide was primarily oncotic. Similar results were obtained with KLA (Supplementary Fig. S1). Inhibition of the antiapoptotic protein Bcl-2 has been shown to enhance chemotherapeutic-induced apoptotic death in prostate cancer cells (36). However, enhanced cell death was not observed when cells were preincubated with a cell-permeable Bcl-2 inhibitor (2 μmol/L; results not shown) for 90 min followed by incubation with 25 μmol/L PTP-KLA for 24 h. To further investigate the mode of cell death, LNCaP cells were treated with different concentrations of KLA and PTP-KLA for 24 h, fixed with 70% ethanol, stained with propidium iodide, and analyzed by flow cytometry. Lower PTP-KLA concentrations (10 μmol/L) induced mainly apoptotic death in a small fraction of LNCaP cells as indicated by a small increase in the sub-G_0 population of cells (Fig. 2*B-i*). However, higher concentrations of PTP-KLA (25 μmol/L) induced oncotic death; Fig. 2*B-ii* indicates a profile characteristic of cells undergoing G_0-phase arrest. DNA laddering assays supported these results (Supplementary Fig. S2). Taken together, these results indicate that whereas low micromolar (<15 μmol/L) concentrations of PTP-KLA and KLA induce apoptosis in LNCaP cells, the percentage of dead/dying cells was significantly lower (<25%) than when high micromolar (>30 μmol/L)

concentrations were used. Use of higher peptide concentrations, however, resulted in oncotic/necrotic death. Finally, LNCaP cells were treated with KLA, PTP-KLA, or equivalent volume of PBS (control) for 24 h, stained with JC-1, and analyzed by flow cytometry to investigate mitochondrial depolarization induced by the KLA peptide (23). The decrease in the red/green fluorescence ratio upon treatment with 30 μmol/L PTP-KLA indicated a significant loss of mitochondrial potential (Fig. 2*C*).

Evaluation of MLN591-KLA (Antibody-Peptide) Conjugates

Conjugation of KLA to anti-PSMA antibody MLN591 enhances its potency in LNCaP cells. In addition to the fusion peptides described above, we investigated MLN591-KLA immunoconjugates as potential site-specific therapeutics for prostate cancer. Briefly, primary amines on the MLN591 antibody were reacted with the hetero-bifunctional cross-linker SPDP to obtain MLN591-SPDP conjugates. The KLA peptide was then conjugated to the activated MLN591 antibody via the thiol group of the COOH-terminal cysteine on the peptide. A ratio of three to five peptide molecules per antibody molecule was consistently obtained using this method.

The antibody-KLA conjugate was significantly more cytotoxic to LNCaP cells than KLA or PTP-KLA. Figure 3*A* compares the cell viability of LNCaP and PC-3 cells when treated with different concentrations of MLN591-KLA for 72 h; no loss of cell viability was observed in either cell line at 24 h. The MLN591-KLA conjugate had no effect on PC-3 cell viability but induced significant cell death in

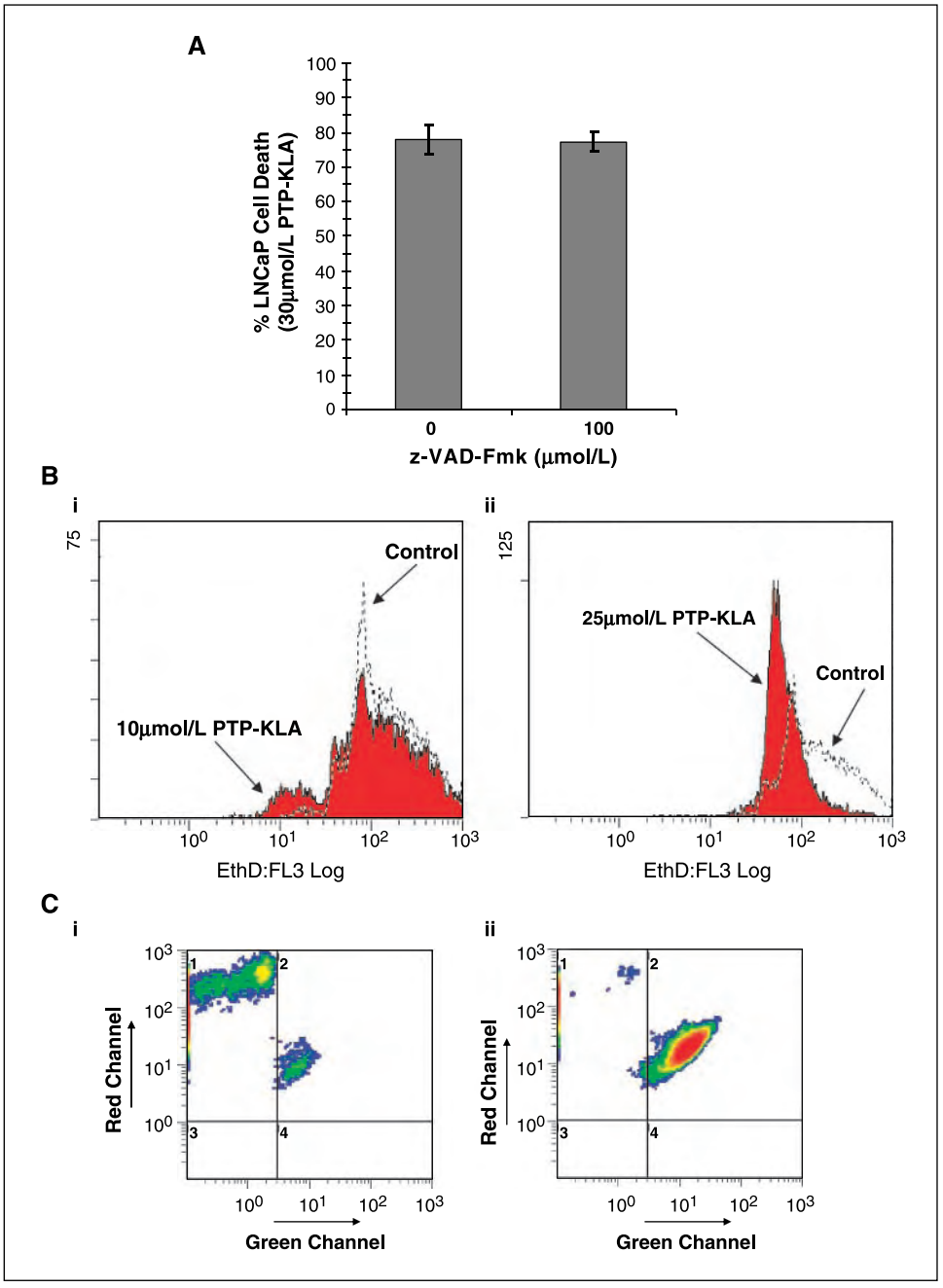

Figure 2. PTP-KLA induces oncotic death in LNCaP cells. *A,* PTP-KLA induces cell death in LNCaP cells via a caspase-independent manner. Cells incubated without and with the pan-caspase inhibitor z-VAD-Fmk (2 h) and then with 30 μmol/L PTP-KLA (22 h). The presence of the caspase inhibitor had no effect on LNCaP cell death. *B,* flow cytometry analysis of LNCaP cells fixed with 70% ethanol and stained with propidium iodide. *i,* low micromolar concentrations (10 μmol/L) of PTP-KLA induce apoptosis in LNCaP cells as seen by the propidium iodide–stained sub-G_0 population. *ii,* higher PTP-KLA concentrations (25 μmol/L) induced necrotic death. *C,* PTP-KLA induces mitochondrial damage in LNCaP cells; following treatment for 24 h, cells were stained with the mitochondria potential indicator dye, JC-1, and subjected to flow cytometry. *i,* a large fraction of cells untreated with PTP-KLA show intact mitochondria as seen by the high ratio of red fluorescent to green fluorescent cells. *ii,* a large fraction of cells treated with 30 μmol/L PTP-KLA undergo mitochondrial damage as seen by the decrease in the ratio of red fluorescent to green fluorescent cells.

LNCaP cells, indicating selectivity of the conjugate toward PSMA-expressing LNCaP cells (Fig. 3*A*). Unconjugated MLN591 did not result in a loss of cell viability in either PC-3 or LNCaP cells. Furthermore, we verified that LNCaP cell viability was unaffected by treatment with equivalent concentrations of either KLA or PTP-KLA for 72 h (data not shown).

Figure 3*B* shows phase-contrast images of the morphologic changes in LNCaP cells and the corresponding fluorescence images showing ethidium homodimer staining. LNCaP cells treated with unconjugated MLN591 (0.4 μmol/L; Fig. 3*B-ii*) exhibit normal morphology, similar to cells treated with PBS (Fig. 3*B-i*), with only a few cells in each field staining with ethidium homodimer. In contrast, cells treated with MLN591-KLA (0.4 μmol/L) exhibit extensive cell death, indicated by rounding, detachment, and extensive staining with ethidium homodimer (Fig. 3*B-iii*).

MLN591-KLA induces apoptosis in LNCaP cells. The homogeneous caspase assay was carried out to investigate caspase activation in LNCaP cells upon incubation with MLN591-KLA for 72 h. Figure 4*A* shows caspase activation when LNCaP cells were treated with MLN591-KLA, whereas those treated with MLN591 exhibited caspase activity comparable to cells treated with PBS. The assay detects caspases 2, 3, and 7, indicating a role for these caspases in MLN591-KLA–mediated cell death. In addition, LNCaP cells were preincubated with 100 μmol/L of the pan-caspase inhibitor z-VAD-Fmk for 2 h before treatment with the MLN591-KLA conjugate. Inhibition of caspase activity with z-VAD-Fmk increased LNCaP viability (Fig. 4*B*), supporting a role for the caspase-mediated apoptotic pathway in MLN591-KLA–induced cell death. Flow cytometry analysis of propidium iodide–stained LNCaP cells after MLN591-KLA treatment indicated that a significant fraction of the cells (>80%) were apoptotic, as indicated by the sub-G_0 population in Fig. 4*C-i*. In contrast, equivalent concentrations of the MLN591 antibody did not induce apoptosis in these cells (Fig. 4*C-ii*). In addition, the MLN591-KLA conjugates induced mitochondrial depolarization in LNCaP cells, which is consistent with the proposed mechanism for cell death induced by the cationic amphipathic peptide KLA (ref. 23; Fig. 4*D*). The mitochondrial integrity of LNCaP cells treated with the unconjugated MLN591 antibody was similar to that of the untreated control.

Comparison of KLA, PTP-KLA, and MLN591-KLA. Dose response studies were employed to determine the *in vitro* LC_{50} values (the concentration required to induce death in 50% LNCaP

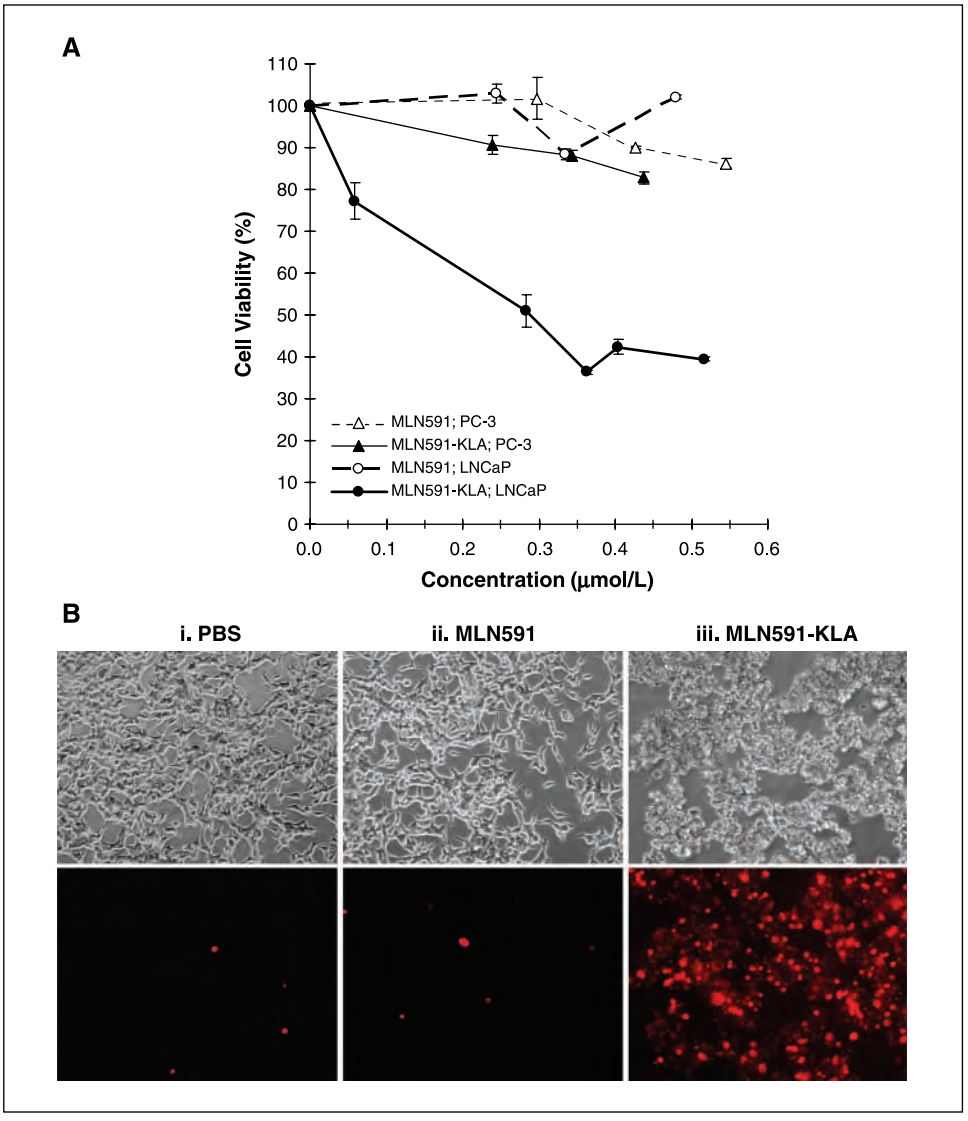

Figure 3. *A,* comparison of MLN591-KLA activity on LNCaP and PC-3 cells. LNCaP and PC-3 cells were treated with different concentrations of MLN591 and MLN591-KLA (3–5 KLA molecules per MLN591 molecule), and cell viability was determined 72 h following incubation and compared with control (cells treated with equal volume of PBS). △, PC-3 cells treated with MLN591; ▲, PC-3 cells treated with MLN591-KLA; ○, LNCaP cells treated with MLN591; ●, LNCaP cells treated with MLN591-KLA. The lines connecting the data points are for visualization only. *B,* phase contrast and fluorescence microscopy images of LNCaP cells treated with PBS (control), MLN591, and MLN591-KLA for 72 h. Cellular morphology (*top*) and ethidium homodimer staining (*bottom*) indicate no cell death upon treatment with (*i*) PBS and (*ii*) 0.4 μmol/L MLN591, but extensive cell death upon treatment with (*iii*) 0.4 μmol/L MLN591-KLA.

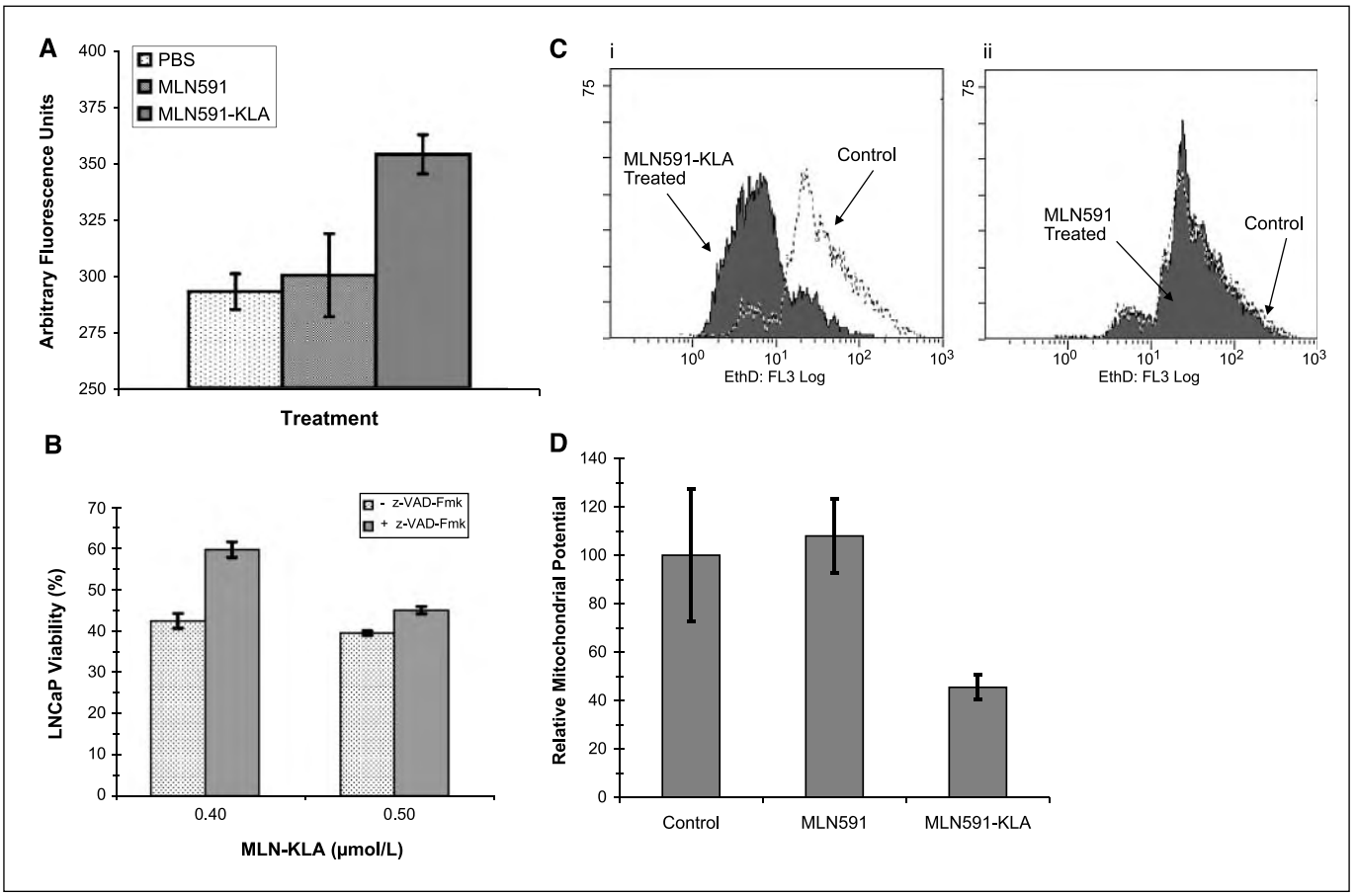

Figure 4. LNCaP death induced by MLN591-KLA is dependent on the caspase-mediated apoptotic pathway. *A,* caspase activation in LNCaP cells treated with PBS, 0.4 μmol/L MLN591, or 0.4 μmol/L MLN591-KLA as determined by the homogeneous caspases assay. LNCaP cells were treated for 72 h as described in Materials and Methods and were incubated with the assay reagents for 5 h. *Columns,* mean of three fluorescence intensity readings (excitation: 485 nm; emission: 510 nm); *bars,* 1 SD. *B,* caspase inhibition results in a reduction of cell death induced by MLN591-KLA–treated LNCaP cells indicating the involvement of the caspase-mediated apoptotic pathway. LNCaP cells were preincubated with the pan-caspase inhibitor z-VAD-Fmk for 2 h followed by the conjugate for 72 h. Cell viability was measured using the MTT assay and compared with the viability of the control (cells treated with an equivalent amount of PBS). *C,* flow cytometry results of LNCaP cells fixed with 70% ethanol and stained with propidium iodide. *i,* treatment with 0.4 μmol/L MLN591-KLA shows a characteristic sub-G_0 population (*solid line* and *solid fill*) when compared with cells treated with the control (equivalent volume of PBS; *dashed line* and *no fill*). *ii,* treatment with MLN591 shows no change in propidium iodide staining characteristics compared with the control. *D,* comparison of JC-1 fluorescence (ratio of fluorescence at 590 nm to fluorescence at 510 nm; excitation: 485 nm) in untreated, 0.45 μmol/L MLN591, and 0.4 μmol/L MLN591-KLA–treated cells. Cells were treated with JC-1 for 45 min, and the fluorescence intensities were measured. The ratio of the fluorescence intensity at 590 nm to that at 510 nm was measured in each case. Relative mitochondrial potential was determined by comparing the ratio of the fluorescence intensities with that of the control. *Columns,* mean of three fluorescence readings; *bars,* 1 SD.

cells in culture) for KLA, PTP-KLA, and MLN591-KLA in LNCaP cells. The LC_{50} value of the MLN591-KLA conjugate for LNCaP cells was 0.28 μmol/L, which is approximately 70-fold lower than that of PTP-KLA (19 μmol/L) and more than two orders of magnitude lower than that of KLA (41 μmol/L), indicating that the anti-PSMA antibody significantly enhances the potency of the KLA peptide (Fig. 5). In addition, whereas PTP-KLA and KLA induced oncotic death in LNCaP cells, MLN591-KLA induced caspase-mediated apoptotic death.

Discussion

A number of chemotherapeutic strategies have been investigated for the ablation of advanced prostate cancer cells (4). Of these, targeted biologicals represent an emerging class of therapeutics that can potentially reduce nonspecific cytotoxicity in collateral organs such as the urethra and bladder, especially in cases of residual disease and secondary metastasis. The transformation of androgen-dependent prostate cancer disease to a highly tumorigenic, metastatic, and androgen-independent phenotype is a result of the accumulation of significant genetic changes (2, 37). Several studies have indicated that the overexpression of antiapoptotic proteins plays a direct role in the abnormal growth of prostatic tissue and correlates with the poor response to radiotherapy (38). For example, overexpression of the antiapoptotic protein Bcl-2 inhibits the mitochondrial pore transition, contributing to the resistance of the transformed cell to apoptosis. Therapeutic strategies that induce mitochondrial depolarization, therefore, are attractive because they can bypass apoptosis resistance mechanisms that act upstream of the mitochondria.

Energy from respiration is stored as a potential and pH gradient across mitochondrial membranes in mammalian cells (chemoosmotic theory; ref. 39). Once internalized, cationic amphipathic molecules preferentially localize at the mitochondria due to the large negative potential gradient across the mitochondrial membrane. Accumulation of a significant concentration of these molecules at the mitochondrial membrane leads to its disruption and, consequently, depolarization, ultimately leading to apoptosis.

We therefore investigated the cationic amphipathic peptide KLA as a potential therapeutic candidate in prostate cancer.

We exploited the overexpression of the PSMA on the surface of prostate cancer cells as means to selectively deliver KLA to these cells. We investigated the potency and mechanism of cell death in human prostate cancer cell lines treated with untargeted, peptide-targeted, and monoclonal antibody–targeted KLA constructs. Peptides targeting the extracellular portion of PSMA have recently been identified from a phage display library and have been shown to possess micromolar (μmol/L) binding affinities to the extracellular portion of the receptor (32). In addition, aptamers (40–42) and monoclonal antibodies (43, 44) have also been used to target the PSMA receptor.

In vitro investigation indicated that the LC_{50} value was 41 μmol/L for untargeted KLA and 19 μmol/L for the targeted PTP-KLA peptide. The use of the PSMA-targeting peptide (PTP) sequence, therefore, modestly enhanced the efficacy (2-fold) of the resulting PTP-KLA fusion compared with the untargeted KLA peptide. Coincubation of PTP-KLA with PTP resulted in lower LNCaP cell death, indicating that the fusion peptide acted by interacting with the PSMA receptor. However, this interaction is in addition to the nonspecific uptake of the fusion peptide mediated by the cell-penetrating activity of the KLA segment.

Contrasting mechanisms of cell death have been proposed for cationic amphipathic peptide-based cancer therapeutics (23, 25, 45, 46). Although some reports indicate that these peptides induce apoptosis due to their ability to depolarize mitochondrial membranes (23, 25, 45), others have reported that these peptides induce plasma membrane lysis leading to oncotic/necrotic death in cancer cells (47). We therefore investigated the mechanism of cell death induced by the peptide- and antibody-targeted KLA constructs.

At concentrations less than 15 μmol/L, PTP-KLA induced apoptotic death in a small fraction of LNCaP cells as indicated by propidium iodide staining/flow cytometry. However, higher concentrations (30 μmol/L) of PTP-KLA induced oncosis in a larger percentage (ca. 80%) of LNCaP cells. These results indicate that plasma membrane permeabilization and cell lysis contribute to necrotic cell death and are similar to those previously reported in the literature (47–49). Thus, in the case of PTP-KLA, the relative affinities of PTP-PSMA interaction and the KLA-plasma membrane interaction may represent competing processes underlying cellular uptake and death. This observation has implications for the design of targeted peptide therapeutics: to engineer fusion peptide therapeutics with high selectivities, the affinity of the targeting peptide (e.g., PTP) must be significantly greater than the plasma membrane lytic activity of the cytotoxic peptide (e.g., KLA). We also verified that the treatment with both KLA and PTP-KLA resulted in mitochondrial depolarization. However, it is not clear whether mitochondrial depolarization was responsible for the cell death induced by KLA and PTP-KLA. It is more likely that the observed depolarization is a consequence of oncosis.

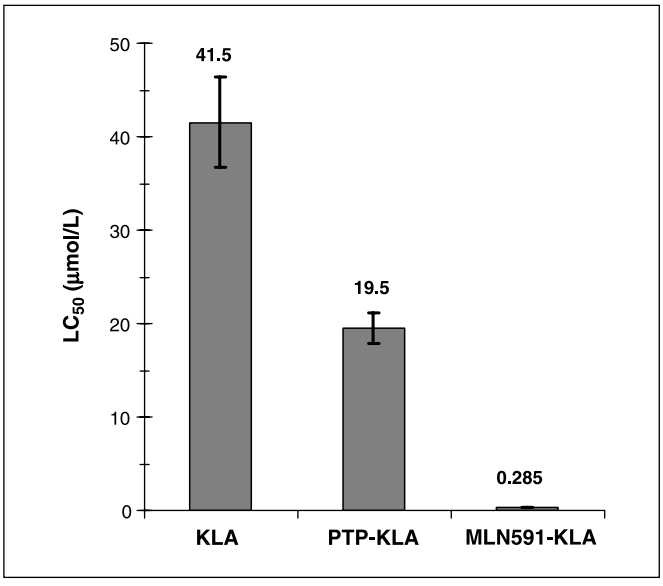

Figure 5. Comparison of the *in vitro* LC_{50} values of untargeted, peptide-targeted, and antibody-targeted fusions/conjugates of KLA with LNCaP cells.

Antibody-based conjugates have been explored as potential therapeutics for a number of malignancies, including prostate cancer. We conjugated KLA to MLN591, a monoclonal antibody that targets the extracellular portion of the PSMA. This antibody has nanomolar affinity for the receptor and can therefore deliver therapeutic cargo with high selectivity to prostate cancer cells that overexpress the receptor. In contrast to the peptides (KLA and PTP-KLA), no cell death was observed when LNCaP cells were treated with the MLN591-KLA conjugate for 24 h. Cells typically entered apoptosis 36 to 48 h after treatment, necessitating measurement of cell viability at 72 h. Significantly lower (submicromolar) concentrations of the MLN591-KLA conjugate were required to induce death in LNCaP cells when compared with PTP-mediated delivery; the antibody enhanced the efficacy of the conjugate by two orders of magnitude compared with the untargeted peptide. Both MLN591 and MLN591-KLA were not toxic to PSMA-negative PC-3 cells under the described experimental conditions. Although the decrease of the LC_{50} of the MLN591-KLA conjugate are in agreement with previous reports on immunoconjugates (45), the exact mechanism by which conjugation of KLA to MLN591 results in the decrease of the LC_{50} value of the MLN591-KLA conjugate is not known. It is hypothesized that the high binding affinity of MLN591 to the receptor results in the localization and subsequently, internalization of KLA molecules inside LNCaP cells. In addition, the presence of a cleavable disulfide bond in the linker region between MLN591 and KLA can play a role in the release of the KLA peptide into the cytoplasm which ultimately results in LNCaP cell death (50).

Investigation of cell death mechanisms confirmed the role of caspase-mediated apoptosis in the ablation of LNCaP cells treated with MLN591-KLA. A significant proportion (>80%) of fixed LNCaP cells stained with propidium iodide showed characteristics of a sub-G_0 population when compared with the control (cells treated with equivalent PBS), indicating that these cells underwent apoptosis. In contrast, cells treated with an equivalent concentration of the unconjugated antibody did not exhibit the presence of a sub-G_0 population, indicating that the antibody alone did not induce apoptosis in these cells. These results are consistent with the cell death results (ethidium homodimer staining) in Fig. 3*B* and indicate that apoptosis is the primary mechanism of cell death induced by MLN591-KLA conjugates. Furthermore, caspase activation was observed in LNCaP cells treated with the MLN591-KLA conjugate. In addition to the enhanced targeting affinity due to the MLN591 antibody, it is possible that steric effects also play a role in preventing cell membrane lysis by the lytic KLA peptide. Conjugation of the (small) lytic peptide to the (large) antibody can keep the former away from the cell membrane, whereas this is not possible in case of the much smaller PTP-KLA fusion peptides. Inhibition of caspase activity resulted in an increase in cell viability,

indicating a role for the caspase-mediated apoptotic pathway. Finally, we verified that the MLN591-KLA conjugate induced mitochondrial depolarization in LNCaP cells after 72 h, in accordance with the previously proposed mechanism of KLA. However, whereas it is tempting to speculate that mitochondrial depolarization is the cause of cell death induced by MLN591-KLA in LNCaP cells, our results do not conclusively prove this. To do so will necessitate the correlation of mitochondrial depolarization kinetics with apoptosis kinetics in LNCaP cells treated with MLN591-KLA, studies that are now ongoing in our laboratory. Future work will also focus on evaluating the antibody-KLA construct *in vivo*, with orthotopically implanted LNCaP tumors in mice.

Conclusions

We have designed, generated, and evaluated targeted fusion peptides and immunoconjugates based on the cationic amphipathic peptide KLA as potential targeted therapeutics for prostate cancer. Our results indicate that antibody-KLA conjugates have promise as potential therapeutics and warrant further investigation. We also investigated the mechanism of cell death induced by these molecular therapeutics. In cases where cationic amphipathic peptides are used, the affinity of the targeting sequence to the receptor must be significantly higher than the membrane-permeabilizing affinity of the lytic peptide to minimize nonspecific death. Thus, whereas lower concentrations of MLN591-KLA are sufficient to induce apoptosis in LNCaP cells due to the enhanced affinity of MLN591 antibody to the receptor, further engineering of peptides targeting the PSMA is required in order for them to be useful in potential therapeutic applications.

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